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**12b. DISTRIBUTION CODE****13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)**

We determined the expression of FGF-2 during steps of mammary ductal dedifferentiation as preliminary investigations in understanding its role in dormancy and relapse of microscopic metastases. Preliminary data suggest that initial low level expression of FGF-2, primarily in myoepithelial cells of normal ducts, is markedly upregulated during ductal hyperplasia, only to be lost with malignant progression. The previously established role of FGF-2 as a morphogenic differentiation agent suggests this to be a reactive process by ductal cells during the uncontrolled proliferation of hyperplasia, an effect largely lost with malignant transformation. More samples will be stained to allow for statistically significant correlations. These data will support a role for FGF-2 expression as a differentiation agent if found in dormant malignant micrometastases in the bone marrow of patients to be stained in this project. To provide mechanistic support, the *in vitro* effects of FGF-2 were determined on breast cancer cell lines. FGF-2 caused a large reduction of well-differentiated breast cancer cells, inhibited clonogenicity in tissue culture and caused massive upregulation of integrin  $\alpha 5$  expression. The ligation of integrin  $\alpha 5$  by fibronectin specifically provided survival signaling and partly restored clonogenicity to the non-growing cells. These experiments provide a paradigm for dormancy.

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## INTRODUCTION

We proposed roles for FGF-2, TGF beta and TGF beta receptor 2 in modulation of growth, survival and dormancy of breast cancer cells that have metastasized to the bone marrow. Before addressing the questions of expression of FGF-2, TGF beta and its beta receptor in the bone marrow micrometastases, we wanted to understand the roles of these growth factors in primary breast cancer cells. Our collaborator in Munich, Germany, Dr. Stephan Braun, who will be staining bone marrow smears containing micrometastases, changed positions and moved to Austria. We felt that the time he needed to collect samples and begin the staining and analysis would be very valuable to permit us to gain some understanding of the roles these proteins played in primary breast cancer cell dedifferentiation and the interaction of breast cancer cells with the bone marrow microenvironment. We began to address these questions using two approaches. Our first approach was to determine the expression of FGF-2 and FGF receptors in mammary ductal epithelial cells during the dedifferentiation steps leading from normal mammary epithelial ducts to invasive, metastatic breast cancer using immunofluorescence antibody labeling. Once the staining procedures are established, we plan to determine TGF beta receptor activation in the same samples. Since we began the project, we learned that staining tissue samples with antibody to phospho-SMAD 2 is a more valid and physiologically relevant approach to determining the effects of TGF beta and the expression of TGF beta receptor 2 than individually staining for the two proteins (1). Our second approach was to determine the effects of FGF-2 on the *in vitro* growth of breast cancer cells on bone marrow stromal proteins and determine the factors that inhibit proliferation and induce survival of these cells as a paradigm for dormancy.

## BODY

### A. Expression of FGF-2 and FGFR1 during mammary duct dedifferentiation

We obtained 4 micron sections of biopsy and mastectomy specimens from the Department of Pathology and Laboratory Medicine of our institution in a blinded manner under an IRB-approved protocol that qualified for exemption because of its blinded retrospective nature. We stained the samples with antibodies to FGF-2 and FGF receptor 1. To date, we stained specimens from 21 patients. Many slides had multiple pathologic features. Table 1 depicts our result to date. While the number of samples stained so far are too few to obtain statistical significance, the data suggest that about half of normal ducts and a third of invasive cancers stain positive with anti-FGF-2 antibody while hyperplasia and carcinoma *in situ* have much greater rates of staining. All of the samples at all stages of dedifferentiation stained positive for FGFR1.

The initial data do not differentiate between epithelial and myoepithelial cells. Unstained slides from these patients as well as additional slides from 50 more patients will be stained with antibodies to FGF-2, phospho-SMAD 2 and cytokeratin 14, a myoepithelial marker, to determine whether the expression of FGF-2 in the various stages of dedifferentiation is found in epithelial or myoepithelial cells and whether expression of FGF-2 correlates with signaling through TGF beta receptors. Statistical correlations using Chi square analysis will determine the significance of these associations and the association between FGF-2 expression and hyperplasia. We recently demonstrated that FGF-2 expression induces expression of TGF beta mRNA and that inhibitory effects of FGF-2 on proliferation are mediated through TGF beta (2). These studies with patient samples will lend support to the biological significance of these findings and will impart a greater understanding to the bone marrow micrometastases staining data once they are available. Results of immunofluorescence staining of representative samples at various stages of dedifferentiation are shown in Figure 1 A and B.

Table 1. Immunofluorescence detection of FGF-2 and FGFR1 in cells of mammary ductal origin

Pathology positive	number of samples	number FGF-2 positive (% positive)	number FGFR positive
normal lobule	7	3 ( 43%)	7
normal duct	10	5 ( 50%)	10
fibrocystic duct	1	1 (100%)	1
typical hyperplasia	2	2 (100%)	2
atypical hyperplasia	2	1 ( 50%)	2
carcinoma in situ	6	4 ( 67%)	6
invasive carcinoma	14	4 ( 29%)	14

Figure 1. Expression of FGF-2 and FGFR1 in mammary duct dedifferentiation

A.

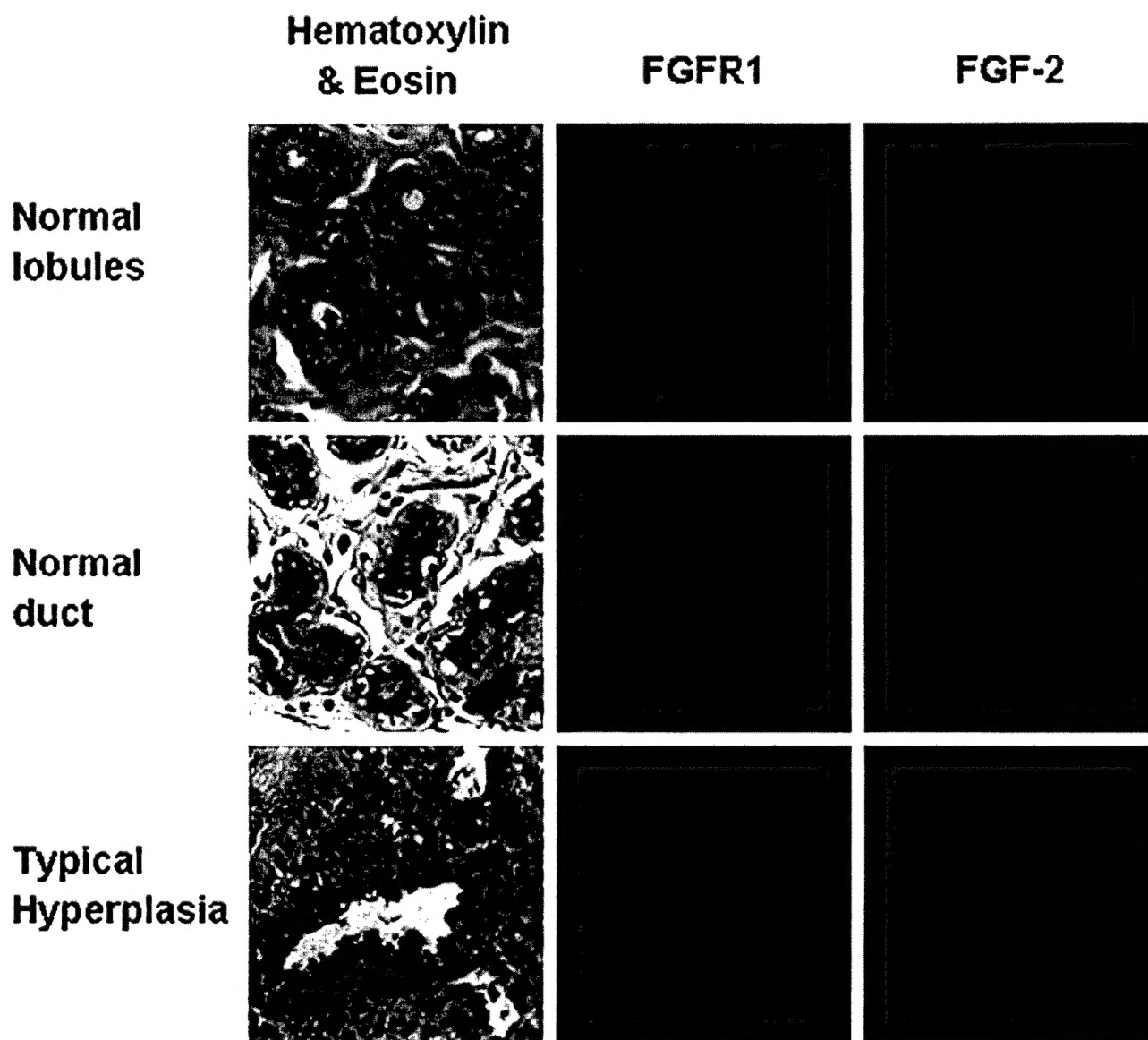
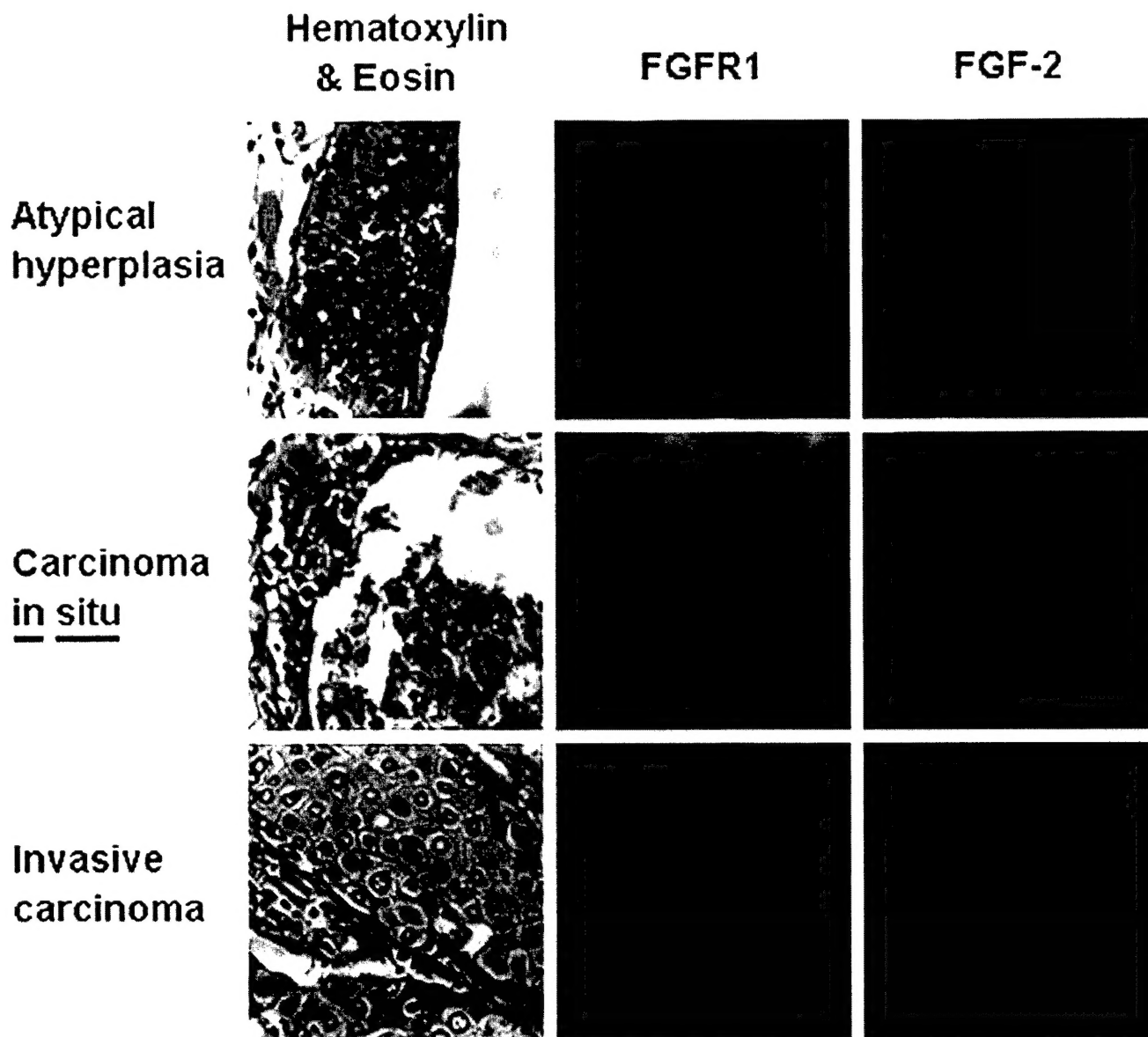


Figure 1.

B.



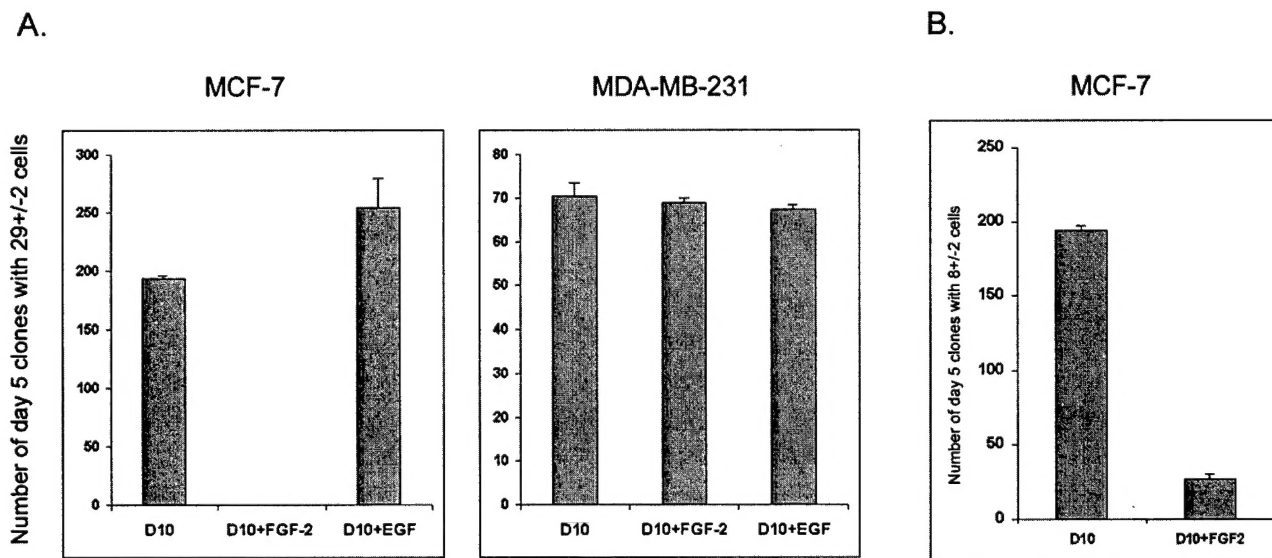
**Figure 1.** Hematoxylin and eosin (H&E) and immunofluorescence staining of mastectomy and biopsy specimens. Slides mounted with 4 micron thick tissue samples were either stained with H&E (left columns) or with primary rabbit antiserum to FGFR1 (Santa Cruz Biotechnology, Santa Cruz, CA) (center column) or monoclonal anti-FGF-2 antibody (Oncogene Research Products, Boston, MA), (right hand columns) and FITC or Texas red-labeled anti-mouse or anti-rabbit secondary antibodies (Santa Cruz), as described (3). Cells were photographed at 100X magnification (H&E) and 400 X magnification (immunofluorescence slides) using an Olympus BX40 fluorescence photomicroscope.

## **B. A role for FGF-2 in the clonogenic potential of breast cancer cells on bone marrow stromal proteins: a paradigm for dormancy**

Breast cancer micrometastases in the bone marrow can remain growth arrested without loss of viability for prolonged periods of time, sometimes up to many years before they enter a state of progressive growth (4). Micrometastatic cells are resistant to chemotherapy and survive in the marrow after multiple cycles of treatment (5). The factors that induce dormancy, that is growth arrest coupled with long-term survival, of occult breast cancer cells in bone marrow microenvironment, and protect the cells from chemotherapy, are largely unknown. Using a panel of breast cancer cell lines we investigated the roles of various stromal proteins and growth factors that are relevant to the bone marrow microenvironment in inducing breast cancer dormancy.

Bone marrow stroma is a rich source of growth factors, that includes basic fibroblast growth factor (FGF-2) (6-10). We and others have shown that FGF-2, a factor implicated in mammary ductal differentiation, induces growth arrest in a variety of relatively differentiated breast cancer cells (11-16). To test the potential role of FGF-2 in inducing growth arrest of breast cancer cells in the bone marrow microenvironment, we initially measured the clonogenic potential of MCF-7, T-47D and MDA-MB-231 breast cancer cells on plastic tissue culture dishes in the presence of FGF-2. The presence of FGF-2, but not EGF, significantly blocked clonogenic growth of relatively well-differentiated MCF-7 and T-47D cells (not shown) but had no effect on the highly dedifferentiated aggressive MDA-MB-231 cells that represent cells that would not be expected to remain dormant once metastatic to the bone marrow (figure 2). The MCF-7 clones that did form in the presence of FGF-2 were arrested in the 8 cell stage. EGF had no effect and served as a negative control.

**Figure 2. FGF-2 inhibits clonogenicity of MCF-7 cells but has no effect on MDA-MB-231 cells**

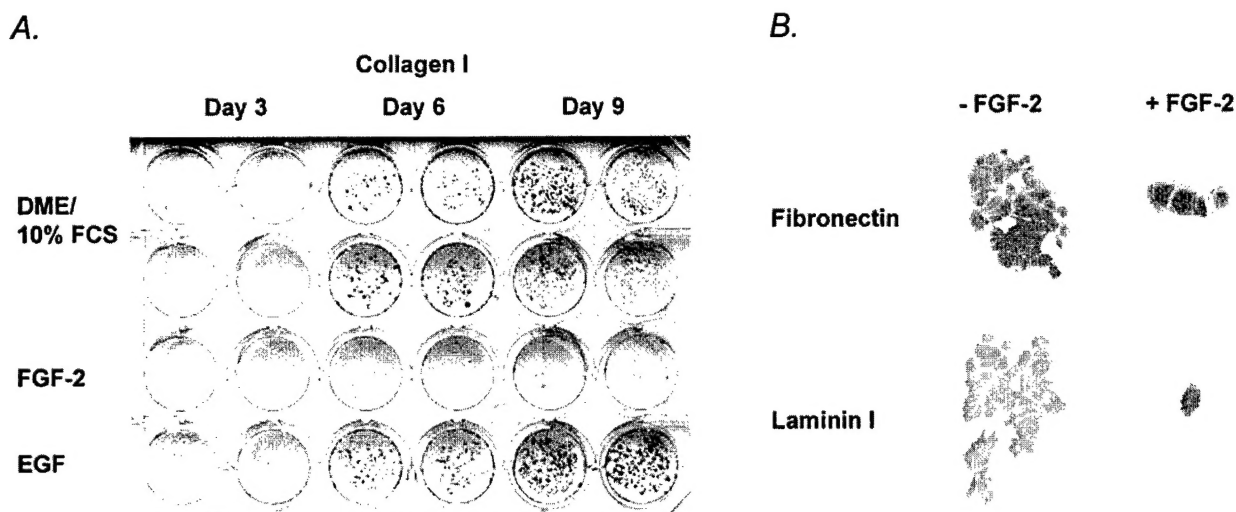


**Figure 2.** One thousand cells per well were incubated in 24 well tissue culture plates with and without the presence of 10 ng/ml basic fibroblast growth factor (FGF-2) or epidermal growth factor (EGF). Plates were stained with crystal violet after a 5 day incubation and clones consisting of A.  $29 \pm 2$  cells and B.  $8 \pm 2$  cells were counted.



MCF-7 and T-47D cells incubated with FGF-2 also had markedly diminished clonogenic potential in colony assays in tissue culture on laminin-, collagen I- and IV-coated and uncoated plates (figures 3 and 4). However, incubation of FGF-2-treated cells on fibronectin-coated plates imparted a survival effect on the colony forming ability of MCF-7 and T-47D cells (not shown) for up to fifteen days assayed.

**Figure 3. Survival effects of fibronectin on FGF-2-inhibited breast cancer cells**

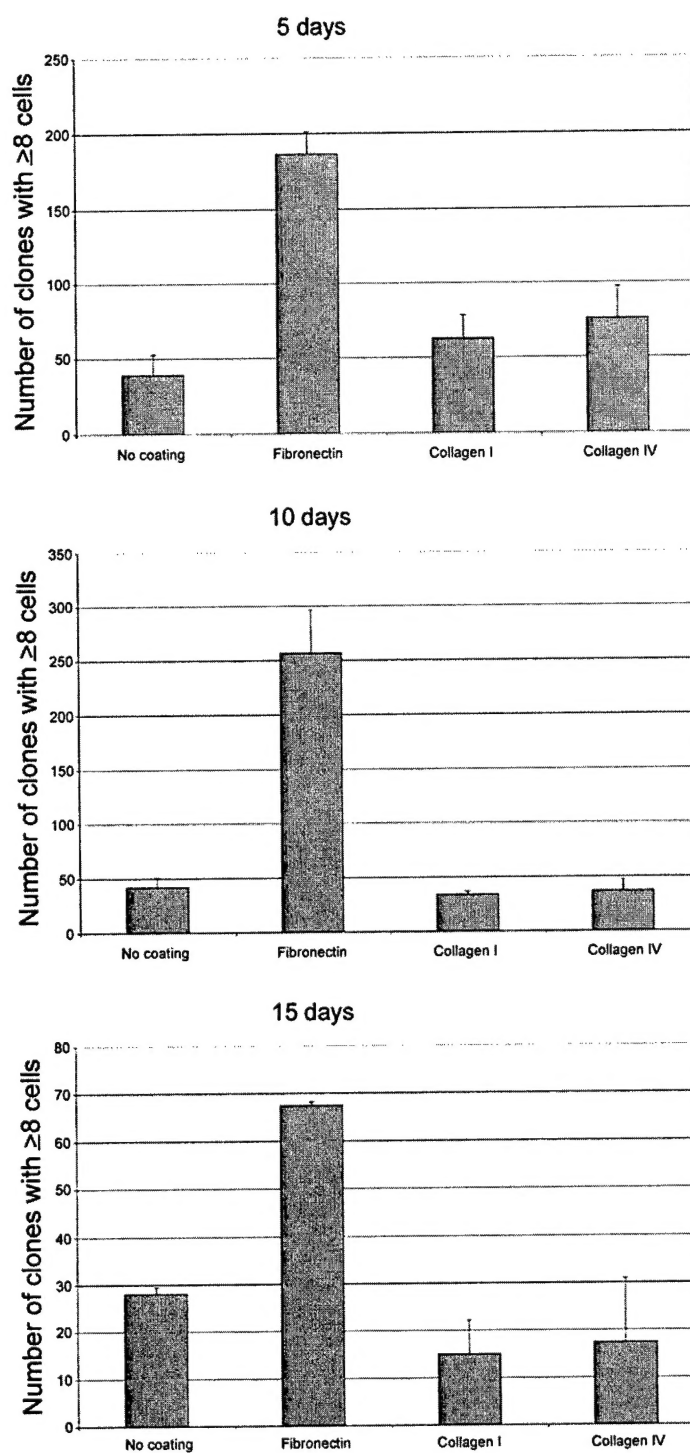


**Figure 3.** MCF-7 cells were incubated at a concentration of 1000 cells/well on 24 well plates coated with **A.** collagen I or **B.** fibronectin or laminin I for variable periods from 3 to 9 days, stained with crystal violet and photographed. **A.** Colony cultures in collagen I-coated dishes demonstrating increased colony formation with time in the two control rows and with 10 ng/ml EGF-treatment and almost complete abolition of colony formation by FGF-2 10 ng/ml. Experiments were done at least twice with similar results. **B.** Similar cellular obliteration was observed on laminin I-coated plates, but incubation on fibronectin yielded survival of a small number of nonproliferating cells. Shown are typical 5 day colonies in control wells and impaired colony formation in wells containing FGF-2 10 ng/ml.



**Figure 4.**

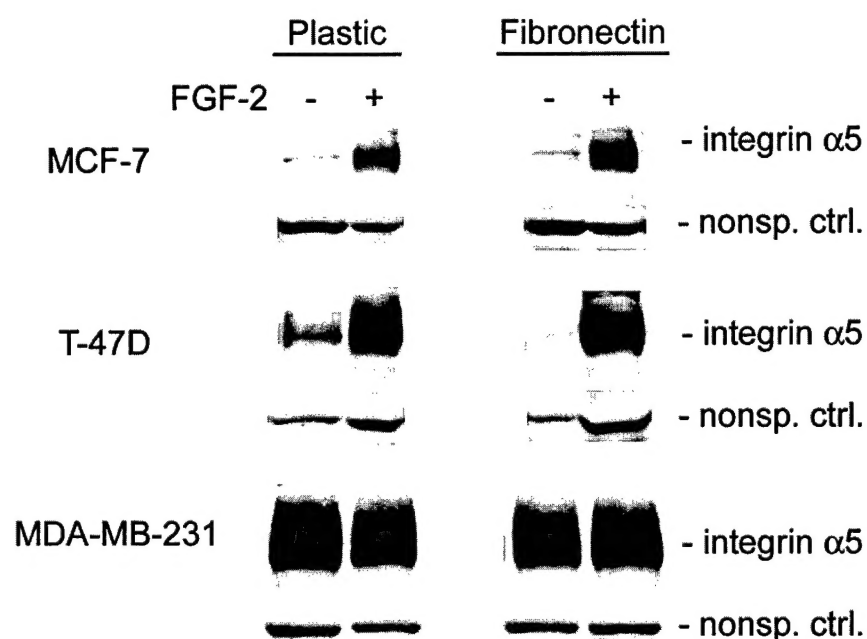
Cloning efficiency of MCF-7 cells in the presence of FGF-2



**Figure 4.** Five thousand MCF-7 cells were incubated per well in duplicate on 6 well tissue culture dishes with various substrata with FGF-2 10 ng/ml. Colonies of 8 cells or greater were counted after staining the plates with crystal violet after 5, 10 and 15 day incubations. Incubation on fibronectin continued to preserve the clonogenicity of these cell lines for up to the 15 days assayed.

Fibronectin is a ligand for integrin  $\alpha 5 \beta 1$  while collagens I and IV and laminin are not. We investigated whether FGF-2 affected the expression of integrin  $\alpha 5$  in the three cells lines. Microarray analysis showed increased expression levels of integrin alpha-5, a fibronectin receptor. Figure 5 is a Western blot demonstrating induction of integrin  $\alpha 5$  expression in MCF-7 and T-47D cells growing on either plastic tissue culture dishes or fibronectin-coated dishes. The increase in integrin  $\alpha 5$  expression was assayed for up to five days and remained sustained. No effect is demonstrated on constitutively very high levels of integrin  $\alpha 5$  in MDA-MB-231 cells. The data suggest an association between unligated integrin  $\alpha 5 \beta 1$  and inhibition of growth and rescue of clonogenic potential by providing a specific ligand for integrin  $\alpha 5 \beta 1$ .

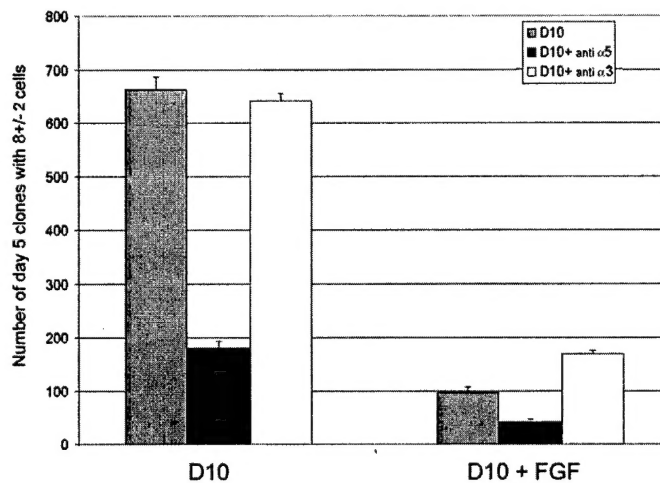
**Figure 5. Induction of integrin alpha 5 expression by FGF-2**



**Figure 5.** Western blot of lysates from cells incubated with FGF-2 10/ng/ml for 3 days on plastic tissue culture dishes or fibronectin coated dishes and stained with anti-integrin  $\alpha 5$  antibody. Nonspecific bands were scanned as loading controls. Experiments were repeated twice and were assayed after 1, 3 and 5 day incubations.

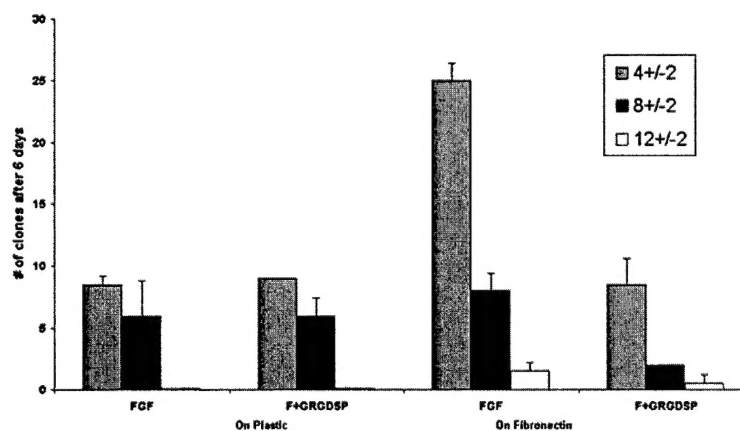
We wanted to determine if the upregulation of integrin  $\alpha 5$  had a physiologic role in clonogenic survival. Antibody to integrin  $\alpha 5$  inhibited the clonogenic potential of MCF-7 cells on fibronectin both with and without FGF-2 treatments (figure 6). Antibody to integrin  $\alpha 3$  was used as a negative control. Blocking peptides that disrupt the interaction of fibronectin with its integrin receptor also reversed the survival effects of fibronectin binding to cells in the presence of FGF-2 (figure 7).

**Figure 6. Integrin  $\alpha 5$ -dependent clonogenic survival of MCF-7 cells on fibronectin**



**Figure 6.** Five thousand MCF-7 cells were incubated per well in quadruplicate on 5-well tissue culture dishes with and without 10 ng/ml FGF-2, in the presence or absence of 2  $\mu$ g neutralizing mouse monoclonal antibody to integrin  $\alpha 5$  or integrin  $\alpha 3$  (Chemicon, Inc, Temecula, CA). Cells were cultured for 5 days, stained with crystal violet and clones with 8+/-2 cells were counted.

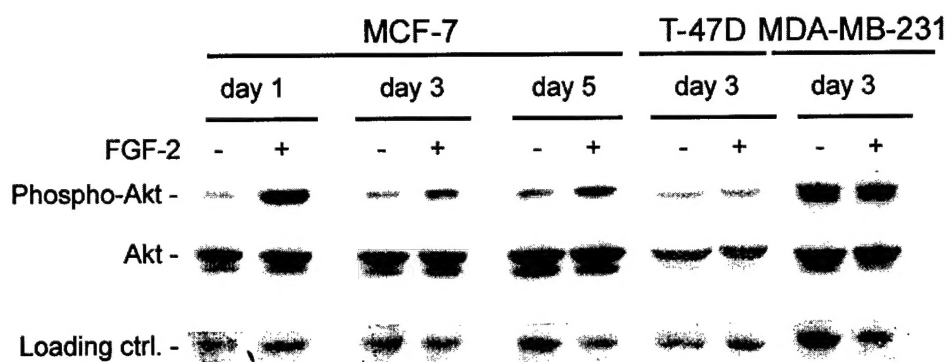
**Figure 7. Fibronectin-specific blocking peptides selectively inhibit clonogenicity on fibronectin.**



**Figure 7.**  $10^3$  MCF-7 (and T-47D, not shown) cells were incubated  $\pm$  fibronectin with 10 ng/ml FGF-2. Fibronectin-blocking peptide GRGDSP 1 ng/ml (American Peptide Co., Inc, Sunnyvale, CA) was added after 3 days and 4, 8 and 12 cell colonies were counted 6 days later. Blocking peptide only inhibited colonies on fibronectin, and not on plastic.

To provide a potential mechanism for survival signaling by integrin  $\alpha 5$  on fibronectin in the presence of FGF-2, initial experiments were conducted to determine the phosphorylation of Akt by FGF-2 in the presence of fibronectin. Figure 8 demonstrates that FGF-2 induced phosphorylation of Akt in MCF-7 and T-47D. Phosphorylation was sustained for the five days of assay. Highly de-differentiated MDA-MB-231 cells, however, express constitutively higher levels of integrin  $\alpha 5$  and phospho-Akt, implicating these molecules in their unlimited growth potential on fibronectin.

**Figure 8. Induction of sustained Akt phosphorylation by FGF-2 on fibronectin**



**Figure 8.** Western blots of lysates from MCF-7, T-47D and MDA-MB-231 cells incubated on fibronectin-coated plates with FGF-2 for up to 5 days were stained with antibody to phospho-Akt or total Akt. Blots show sustained phosphorylation of Akt by FGF-2 in MCF-7 and T-47D cells but no effect on constitutive Akt phosphorylation in MDA-MB-231 cells. No effect was noted on total Akt levels. Stained membrane was used as a loading control.

## KEY RESEARCH ACCOMPLISHMENTS

1. FGF receptor 1 is expressed in all stages of mammary duct dedifferentiation to cancer – preliminary data.
2. FGF-2 expression is markedly upregulated in ductal hyperplasia – preliminary data.
3. FGF-2 inhibits survival and clonogenic potential of well-differentiated breast cancer cells in vitro.
4. FGF-2 markedly upregulates integrin  $\alpha 5$  in well-differentiated breast cancer cell lines.
5. Ligation of integrin  $\alpha 5$  by fibronectin specifically imparts survival signaling and partially restores clonogenicity of FGF-2-treated cells.

## REPORTABLE OUTCOMES

Boots M, Korah R, and Wieder R. Fibronectin receptors promote survival of growth-arrested breast cancer cells on stromal proteins: a paradigm for breast cancer dormancy in bone marrow. (2002) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, p. 95, #68.

## CONCLUSIONS

1. We and others have demonstrated that FGF-2 is a morphogenic differentiation agent that functions in mammary ductal differentiation, and is lost with malignant transformation. Our preliminary observations show that FGF-2 expression is markedly upregulated in ductal hyperplasia and confirm that it is lost with malignant transformation. These observations suggest an attempted response to the loss of control of proliferation by the ductal epithelial cells by expression of a morphogenic differentiation agent. This response appears lost for the most part as the malignant process progresses. These observations will be correlated with the expression of FGF-2 in bone marrow micrometastases and dormancy.
2. Our data suggest that stromal proteins in the bone marrow microenvironment, such as fibronectin, may provide protection of metastatic cancer cells from cell death induced by physiologic factors in the bone marrow microenvironment and from exogenous toxicity such as chemotherapy or radiation therapy. The ability to disrupt the interaction between fibronectin/integrin  $\alpha 5 \beta 1$  with blocking antibodies to integrin  $\alpha 5$  and  $\beta 1$  (experiments in progress), peptides to the fibronectin binding site, antisense phosphorothioated oligonucleotides to integrins  $\alpha 5$  or  $\beta 1$  (experiment in progress), can result in disruption of the survival signal initiated by fibronectin/integrin  $\alpha 5 \beta 1$  interaction and thereby become sensitive to chemotherapy and radiation therapy or other biologic therapy-mediated cell death. This approach may sensitize both well-differentiated cells that are non-cycling and dormant in the bone marrow that receive survival protection from ligation to fibronectin in the microenvironment and highly de-differentiated cells that are actively proliferating in the bone marrow that also receive survival signaling from interaction with fibronectin through a constitutively upregulated integrin  $\alpha 5$ .

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## APPENDICES

none